* NOTICES *



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CLAIMS

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[Claim(s)]
[Claim 1]Following steps (a) An extraction refining method of RNA containing - (c).
(a) By adding, mixing or contacting a solution which contains a chaotropic material in living thing materials, such as a cell, an extract which consists of organic solvents, and a nucleic acid unity solid phase carrier under an acid condition, RNA is made eluted by an eluate from solid phase which washed a solid phase carrier to which made RNA contained in living thing material adsorb on solid phase, and RNA was made to stick at the (b) above-mentioned (a) process with a penetrant remover, and was washed at the (c) above-mentioned (b) process.
[Claim 2]An extraction refining method of the RNA according to claim 1 whose acid conditions are pH 3-6.
[Claim 3]An extraction refining method of the RNA according to claim 1 whose pH of a solution containing a chaotropic material is 3-6.
[Claim 4]An extraction refining method of the RNA according to claim 1 whose chaotropic material is a guanidine thiocyanate.
[Claim 5]An extraction refining method of the RNA according to claim 1 whose organic solvents are water saturation phenol or buffer solution saturated phenol, chloroform, or such combination.
[Claim 6]An extraction refining method of the RNA according to claim 1 which is a carrier in which a nucleic acid unity solid phase carrier contains silica.
[Claim 7]An extraction refining method of the RNA according to claim 1 whose nucleic acid unity solid phase carriers are particles.
[Claim 8]An extraction refining method of the RNA according to claim 1 which is the particles in which a nucleic acid unity solid phase carrier contains a superparamagnetism metallic oxide.
[Claim 9]An extraction refining method of the RNA according to claim 1 whose extract is water or TE buffer.
[Claim 10]An extraction refining method of the RNA according to claim 1 a nucleic acid unity solid phase carrier's being a carrier containing a superparamagnetism metallic oxide, and including further a process of separating a nucleic acid unity solid phase carrier and the liquid phase using magnetism.
[Claim 11]An extraction refining reagent kit of RNA containing an extract, a nucleic acid unity solid phase carrier, a penetrant remover, and an eluate which consist of a solution, pH three to 6 buffer solution, and an organic solvent containing a chaotropic material.

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## DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention]This invention relates to the reagent kit for carrying out extraction refining of the RNA which uses RNA for the method of purity improving extraction refining simple, and this method from living thing materials, such as a cell, using a nucleic acid unity solid phase carrier. This reagent kit can be applied also to automatic nucleic acid extractor.
[0002]
[Description of the Prior Art]Extraction refining of the nucleic acid from living thing materials, such as a cell containing nucleic acid, is an important step in gene engineering or the field of a clinical diagnosis. For example, when it is going to analyze about a certain gene, it is required to extract nucleic acid, such as DNA and RNA, from living thing materials, such as a cell holding the gene, first. Also in the DNA/RNA diagnosis for detection of infectious agents, such as bacteria and a virus, to detect is required, after extracting bacteria and the nucleic acid of a virus from living thing materials, such as blood. Generally, nucleic acid called DNA and RNA which are contained in living thing material does not necessarily exist in the state where it separated, and exists in the husks of the cell membrane which comprises protein, lipid, and sugar, a cell wall, etc., and, in almost all cases, the nucleic acid itself forms the complex with protein. Therefore, in carrying out extraction refining of the nucleic acid from living thing material. The physical crushing treatment according to an ultrasonic wave or heat first, and the enzyme treatment by protease, It is necessary to separate nucleic acid and the column chromatography etc. which use carriers, such as extract operation by organic solvents, such as phenol, ultra-centrifugal separation, an ionic exchanger, need to refine nucleic acid out of debris further by performing processing by the surface-active agent or a denaturing agent, etc. These techniques are together put according to the use of nucleic acid, or the charge of a start material and the nucleic acid extracted further, are optimized, respectively, and are used.
[0003]As a method of carrying out extraction refining of the RNA from living thing materials, such as a cell, what is called an AGPC method [Analytical Biochemistry 162 and 156-159] (1987) is generally used well. This method into living thing materials, such as (1) cell, a guanidine thiocyanate and phenol, Add the solution containing chloroform one by one, and a cell membrane and a cell wall are destroyed, Denature the protein combined with nucleic acid and genomic DNA is made to distribute to an organic phase further, (2) separating only the aqueous phase in which RNA is contained by centrifugal separation -- (3) -- making RNA insolubilize (an ethanol sedimentation method or an isopropanol sedimentation method), and by adding ethanol or isopropanol to this aqueous phase, (4) It is a method using making centrifugal separation separate only RNA further. This AGPC method has a short time and the strong point in which RNA is obtained simple, as compared with the RNA extraction purification method using other ultracentrifugation. However, since the step which requires a long time called the complicated operation of centrifugal separation or separation of the aqueous phase, and an ethanol sedimentation method or an isopropanol sedimentation method is required for this method, When many samples, such as a clinical diagnosis, need to be analyzed especially promptly, simpler and the method of carrying out extraction refining of the RNA for a short time are required.
[0004]There is the method of on the other hand using silica as a nucleic acid unity solid phase carrier as a simple nucleic acid extraction method [JP,H2-289596,A]. Since this method can extract nucleic acid from living thing materials, such as a cell, by a single step and also low-concentration buffer solution, such as

water or TE buffer, is used for it as an eluate, There is an advantage that the extracted nucleic acid can be promptly used for next analysis directly without passing through the operation for demineralization of an ethanol sedimentation method etc. and concentration. However, when extraction of RNA is tried from a cell by this method, in order that genomic DNA as well as RNA may stick to a silica carrier, into recovering liquid, a lot of genomic DNA mixes besides RNA. Therefore, in order to obtain only RNA, it is indispensable to perform refining operation of enzyme treatment, ultra-centrifugal separation, column chromatography, etc., etc. further.

[0005]

[Problem(s) to be Solved by the Invention]The purpose of this invention is to solve the above-mentioned problem of the art which exists from the former, and is extracting RNA by the short time and a high grade, and providing the method of refining, without needing complicated operation for RNA from living thing materials, such as a cell.

[0006]

[Means for Solving the Problem]In order to solve an aforementioned problem, as a result of inquiring wholeheartedly, by solution which has suitable pH, organic solvent, and a nucleic acid unity solid phase carrier, this invention persons found out that extraction refining of the RNA could be carried out simple from living thing material, and reached this invention.

[0007]That is, this invention is an extraction refining method of RNA containing following-steps (a) - (c). (a) By adding, mixing or contacting a solution which contains a chaotropic material in living thing materials, such as a cell, an extract which consists of organic solvents, and a nucleic acid unity solid phase carrier under an acid condition, RNA is made eluted by an eluate from solid phase which washed a solid phase carrier to which made RNA contained in living thing material adsorb on solid phase, and RNA was made to stick at the (b) above-mentioned (a) process with a penetrant remover, and was washed at the (c) above-mentioned (b) process.

[0008]In this invention, nucleic acid unity solid phase carriers are the particles containing a superparamagnetism metallic oxide, and a process of separating a nucleic acid unity solid phase carrier and the liquid phase further using magnetism may be included.

[0009]This invention is an extraction refining reagent kit of RNA containing an extract, a nucleic acid unity solid phase carrier, a penetrant remover, and an eluate which consist of a solution, pH three to 6 buffer solution, and an organic solvent containing a chaotropic material.

[0010]

[Embodiment of the Invention]The extraction refining method of RNA by this invention is roughly divided into the three-stage of (a) dissolution and an adsorption process, the (b) washing process, and (c) elution process.

[0011](a) Dissolve living thing material and make RNA contained in living thing material stick to nucleic acid unity solid phase in the dissolution and an adsorption process by adding, mixing or contacting lysis liquid, an organic solvent, and a nucleic acid unity solid phase carrier into living thing materials, such as a cell, under an acid condition. It may add independently into living thing materials, such as a cell, or the above-mentioned solution, an organic solvent, and a nucleic acid unity solid phase carrier may be added simultaneously. In this invention, the solution containing a chaotropic material, the extract which consists of organic solvents, and a nucleic acid unity solid phase carrier are set to pH 3-6, and it is preferably characterized by making it add, mix or contact in the pH 4 neighborhood still more preferably under an acid condition.

[0012]As a living thing material used in this invention, body fluid other than tissue, a cultured cell, and a bacterial culture thing, such as constituents of blood, such as whole blood, a blood serum, leucocytes, saliva, urine, and sperm, is mentioned, for example.

[0013]It is preferred to make the solution containing the chaotropic material used in this invention contain a buffer. This may be added as buffer solution, even if beforehand contained in the solution, and after dissolving a cell. As this buffer, if generally used, it will not be limited in particular, but what has buffer capacity in one pH of pH three to 6 ranges is more preferred. For example, sodium acetate acetic acid, sodium acetate chloride, etc. are mentioned, and the range of 3-6 is preferred for 1 - 500mM and pH as the operating concentration.

[0014]A chaotropic material is contained in the solution used in this invention. It has the operation which makes the water solubility of a hydrophobic molecule which is generally known as a chaotropic material

increase as this chaotropic material, and it will not be limited especially if it contributes to the combination to the solid phase of RNA further. Specifically, a guanidine thiocyanate, guanidinium hydrochloride, iodine sodium, iodine potassium, sodium perchlorate, etc. are mentioned. The large guanidine thiocyanate of the inhibition effect over RNase which decomposes RNA is used preferably among these. As for the operating concentration of these chaotropic materials, when it changes with chaotropic materials used, for example, uses a guanidine thiocyanate, it is preferred to use it so that it may become the range of 3-5.5M.

[0015]The solution containing a chaotropic material may be made to contain a surface-active agent in order to denature the protein contained in destruction or the cell of a cell membrane. As this surface-active agent, if generally used for the nucleic acid extraction from a cell etc., will not be limited in particular, but specifically, Polyoxyethylene octylphenyl ether, polyoxyethylene sorbitan monolaurate, Nonionic surface active agents, such as polyoxyethylene sorbitan monooleate, A dodecyltrimethylammonium bromide, dodecyl trimethylammonium chloride, Ampholytic surface active agents, such as anionic surfactants, such as cationic surfactants, such as Sept Iles trimethylammonium bromide, sodium dodecyl sulfate, N **RAU roil sarcosine sodium, and sodium cholate, and phosphatidylethanolamine, are mentioned. N-lauroyl sarcosine sodium is used preferably among these. As for the operating concentration of these surface-active agents, when it changes with surface-active agents used, for example, uses N **RAU roil sarcosine sodium, it is preferred to use it so that it may become 0.1 to 2% of range.

[0016]If the combination to the solid phase of RNA is not barred and the combination to the solid phase of DNA is barred as an organic solvent used in this invention, it will not be limited in particular.Although the details about this principle are unknown, by adding an organic solvent to the liquid phase, I lower the polarity of the liquid phase moderately and think that the selectivity of combination with solid phase is given to RNA and DNA from which the polarity on the surface of a molecule differs by that. As an example of the organic solvent used in this invention, they are water saturation phenol, buffer solution saturated phenol, chloroform, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, and the 3-methyl- 1. - Propanol, acetone, etc. are mentioned. What mixed chloroform with water saturation phenol or water saturation phenol at a suitable rate is [ among these ] preferred.

[0017]If it is a carrier which has a hydrophilic surface which can hold nucleic acid by adsorption, i.e., a reversible combination, under existence of chaotropic ion as a nucleic acid unity solid phase carrier used in this invention, it will not be limited in particular. Specifically, a silica dioxide, i.e., silica, is used preferably. If it seems that the reversible combination with the above nucleic acid is not barred, a complex with what performed the surface treatment by chemical modification, and other substances, such as a superparamagnetism metallic oxide, is also contained in other substances which comprise silica, for example, glass, diatomite, or these. As a gestalt of this nucleic acid unity solid phase carrier, especially although particles, a filter, a reaction vessel, etc. are mentioned concretely, it is not limited. The gestalt of the grain child in consideration of the efficiency of adsorption and elution is [ among these ] more preferred, and 0.05-500 micrometers is more preferred for particle diameter at this time.

[0018](b) A washing process is a process of separating only the nucleic acid unity solid phase carrier to which RNA stuck as much as possible from the mixture of the living thing material in the above (a), a solution, an organic solvent, and a nucleic acid unity solid phase carrier. At this time, it is preferred to wash about about 1 to 3 times repeatedly using a penetrant remover.

[0019]As concrete separating mechanism of the liquid phase and the solid phase in this invention, it changes with gestalten of the nucleic acid unity solid phase carrier to be used, and when a nucleic acid unity solid phase carrier is a gestalt of particles, centrifugal separation, filtration, column operation, etc. are preferred. If that in which the superparamagnetism metallic oxide was included is used as a solid phase carrier in particles, the simple magnetic separation using a magnet etc. becomes possible, and it is more suitable.

[0020]As a penetrant remover used in this invention, the elution of the plasmid DNA from a solid phase carrier is not promoted, and especially if the combination to the solid phase of genomic DNA or protein is barred, it will not be limited. A 3 - 5.5M guanidine thiocyanic acid solution or 40 to 100% ethanol is preferred, and if these penetrant removers are used together, specifically, it is more suitable. That is, it is preferred to wash by ethanol further 40 to 100% first, after a guanidine thiocyanic acid solution washes. If the lysis liquid and the organic solvent which were first used in the dissolution and an adsorption process are used as a penetrant remover, it is effective by removal of genomic DNA and protein. At this time, it is

preferred to wash by ethanol 40 to 100% continuously.
[0021](c) An elution process is a process which makes this RNA elute from the nucleic acid unity solid phase carrier to which RNA in the above-mentioned (b) process stuck. Therefore, as an eluate used in this invention, if the elution of RNA from solid phase is promoted, it will not be limited in particular. Specifically, water or TE buffer [10mM trischloride buffer solution, 1mMEDTA, pH 8.0] is preferred. RNA collected at this time can be directly used for the enzyme reaction which uses reverse transcriptase etc., without performing demineralization of dialysis, an ethanol sedimentation method, etc., and concentration operation.

[0022]Since the extraction refining method of RNA by this invention comprises a simple process, it can be easily applied to the nucleic acid extracting apparatus which automated the separating operation and reagent dispensation operation of solid phase.
[0023]The extraction refining reagent kits of RNA of this invention contain pH three to 6 solution containing a chaotropic material, the extract which consists of organic solvents, a nucleic acid unity solid phase carrier, a penetrant remover, and an eluate.
[0024]
[Example]Hereafter, although working example explains this invention still more concretely, this invention is not limited to these working example.
The preparation HeLa cells of the extraction refining (1) HeLa cell of RNA from working example 1 HeLa cell were cultured for 37 ** and four days by 15 ml of 10% fetal-calf-serum (made by Gibco BRL) content Dulbecco's-modified-Eagle's-medium (made by NISSUI) culture media. The cell separated by trypsinization is moved to 15-ml ********** after the end of culture, After centrifuging for 5 minutes and removing 1,000 rpm of supernatant liquid, it was suspended by 10-ml PBS [137mM sodium chloride, 2.7mM potassium chloride, 4.3mM disodium hydrogenphosphate, and 1.4mM potassium dihydrogen phosphate (pH 7.4)]. When the cell number was measured about this, it was a $7x10^7$ individual. This was poured distributively to the 1.5-ml ** micro tube so that the cell number per tube might serve as a $1x10^6$ individual, it centrifuged for 5 minutes and 3,000 rpm of cell pellets obtained by removing supernatant liquid were used as extraction material.
[0025]Into the cell prepared by the extraction refining above (1) of RNA, (2) Lysis liquid [4M guanidine thiocyanic acid of 500microl, 25mM sodium acid citrate (pH 7.0), 0.5%N-lauroyl sarcosine sodium, Add 0.1M 2-mercaptoethanol] and it was made to dissolve, and water saturation phenol of 2M sodium acetate acetic acid [ of 50microl ] (pH 4.0) and 500 moremicrol was added continuously, and it mixed violently. To this, the suspension of the 0.5g [/ml ] magnetic silica particle (the particle diameter of 1-10 micrometers, tri-iron tetraoxide particle 30% content, specific surface area $280m^2/g$, 0.025ml [ // g ] and pore volume surface pore-diameters [ of 2-6 nm ]:Suzuki fats-and-oils company make) of 40microl was added, and it mixed for 10 minutes at the room temperature to it. Next, the micro tube was installed in the magnetic stand (MPC-M : made by a dynal company), magnetic silica particles were collected, and supernatant liquid was removed. After having removed the micro tube from the magnetic stand, adding a 1-ml penetrant remover [5.3M Guanidine thiocyanic acid and 52mM tris-chloride (pH 6.4)] and fully mixing, particles were washed by installing in a magnetic stand similarly and removing supernatant liquid. Similarly, it washed by 1 ml of 70% ethanol, and with a 1-ml penetrant remover, again, it continued, and particles were washed and particles were washed twice once by ethanol 100%. After removing supernatant liquid, a micro tube is installed on the heat block set as 55 **, by neglecting it for 20 minutes, evaporative removal of the ethanol in a tube was carried out, and particles were dried. After adding the sterilized water of 100microl to this and mixing for 10 minutes at a room temperature, it installed in the magnetic stand, magnetic silica particles were collected, and supernatant liquid was collected. The amount of recovering liquid was about 80microl.
[0026]Agarose gel electrophoresis is presented with 10microl among recovering liquid, and the result a photograph of was taken is shown in drawing 1 (lane 1) after ethidium bromide dyeing. In the RNA sample extracted by the method of this invention, most mixing of genomic DNA was not accepted but it has checked that RNA could improve [ purity ] extraction refining by the method of this invention so that clearly from drawing 1 (lane 1).
[0027](3) Detection of RNA in recovering liquid was tried to the recovering liquid obtained by the detection above (2) of Homo sapiens transferrin receptor RNA by RT-PCR by performing RT-PCR for Homo sapiens transferrin receptor RNA at a target. RT-PCR was performed using commercial reagent kit RT-PCR high

(made by Toyobo Co., Ltd.), and the primer for the Homo sapiens transferrin receptor amplification (made by CL5407-1:Clontech). First, M-MLV reverse transcriptase and the reagent for reverse transcription containing the primer for reverse transcription were added to 10microl among the recovering liquid obtained above (2), the last volume was set to 20microl, this was kept warm for 20 minutes 42 **, and the reverse transcription reaction was performed. Operation same for not adding reverse transcriptase in parallel was performed, and this was considered as negative control of the reverse transcription reaction. Next, the reagent for PCR which contains heat-resistant DNA polymerase in the reaction mixture after reverse transcription is added, The last volume was set to 100microl, for 95 ** and 1 minute, for 56 ** and 1 minute, and for 72 ** and 1 minute were carried out 30 cycles in DNA Thermal Cycler (made by Perkin Elmer Cetus), and PCR was performed. Agarose gel electrophoresis is presented with 10microl among reaction mixture, and the result a photograph of was taken is shown in drawing 2 after ethidium bromide dyeing. The size marker in which the lane 1 consists of a PstI digest of random phage DNA among a figure, The migration pattern of the RT-PCR amplification products of RNA by which extraction refining was carried out by the method of showing the lane 2 in working example 1, and the lane 3 show the migration pattern of a PCR amplification product when negative control of a reverse transcription reaction is used. It has checked that amplification products are looked at by only the reaction mixture (lane 2) which performed the reverse transcription reaction, and extraction of RNA was possible and it could be promptly used for the analysis by RT-PCR by the method of this invention so that clearly from drawing 2.

[0028]The blood serum with which HCV of the extraction refining (1)$1x10^7$ copy / ml of working example 2 hepatitis-C-virus (HCV) RNA is contained was diluted with the negative blood serum, the dilution series of a $2x10^5$ - $2x10^3$ copy / ml was built, and these were made into extraction material. Blood serum sample 50microl (an equivalent for $1x10^4$ - a $1x10^2$ copy) of each dilution series was used, and RNA was extracted by the same method as working example 1.

[0029](2) Detection of HCV-RNA in recovering liquid was tried to the recovering liquid obtained by the detection above (1) of HCV-RNA by RT-PCR by performing RT-PCR for the untranslation region of HCV-RNA at a target. RT-PCR was performed using commercial reagent kit RT-PCR high (made by Toyobo Co., Ltd.). First, M-MLV reverse transcriptase and the reagent for reverse transcription containing the primer for reverse transcription were added to 5microl among the recovering liquid obtained by (1), the last volume was set to 20microl, this was kept warm for 60 minutes 42 **, and the reverse transcription reaction was performed. Next, the reagent for PCR which contains heat-resistant DNA polymerase in the reaction mixture after reverse transcription is added, Set the last volume to 25microl and in DNA Thermal Cycler (made by Perkin Elmer Cetus). First, two steps of PCR were performed by 38 cycles' adding the reagent for PCR for for 94 ** and 30 seconds, for 53 ** and 30 seconds, and for 72 ** and 1 minute further, setting the last volume to 30microl, and carrying out for 94 ** and 30 seconds, for 50 ** and 1 minute, and for 72 ** and 1 minute 28 cycles. Agarose gel electrophoresis is presented with 10microl among reaction mixture, and the result a photograph of was taken is shown in drawing 3 after ethidium bromide dyeing. The lane 1 is a migration pattern of the RT-PCR amplification products of RNA by which extraction refining was carried out by the size marker which consists of a PstI digest of lambda phage DNA, and the method which the lanes 2-7 show to working example 2 among a figure.

The lane 2 and the lane 3 show a result when a $1x10^4$ copy, the lane 4, and the lane 5 use the blood serum sample in which a $1x10^3$ copy, the lane 6, and the lane 7 contain HCV of a $1x10^2$ copy.

Amplification products are seen about the blood serum sample containing HCV of a $1x10^4$ copy and a $1x10^3$ copy, and extraction of HCV-RNA from a blood serum sample is possible by the method of this invention so that clearly from drawing 3, It has checked that it could be promptly used for the analysis by RT-PCR.

[0030]In order to check that purity can improve [ RNA ] extraction refining as compared with comparative example 1 conventional method, extraction of RNA was tried with the conventional method using a chaotropic material and a silica particle.

Into the cell prepared in working example 1 (1), lysis liquid [4.7M GUANISHI gin thiocyanic acid of 900microl, Add 46mM trischloride (pH 6.4), 1.2% polyoxyethylene octylphenyl ether, and 20mM EDTA], and it was made to dissolve, and the 0.5mg [/ml ] magnetism silica suspension of 40microl was added continuously, and it mixed for 10 minutes at the room temperature. Next, the micro tube was installed in

the magnetic stand, magnetic silica was collected, and supernatant liquid was removed. Subsequently, they are 2 time and 70% of one ml like the method of working example (2) at 1-ml penetrant remover [5.3M guanidine thiocyanic acid and 52mM trischloride (pH 6.4)]. With ethanol Particles were washed once [ ] by ethanol 2 times and 100%. After removing supernatant liquid, a micro tube is installed on the heat block set as 55 **, by neglecting it for 20 minutes, evaporative removal of the ethanol in Chubb was carried out, and particles were dried. After adding the sterilized water of 100microl to this and mixing for 10 minutes at a room temperature, it installed in the magnetic stand, magnetic silica particles were collected, and supernatant liquid was collected. The amount of recovering liquid was about 80microl. Agarose gel electrophoresis is presented with 10microl of the recovering liquid, and the result a photograph of was taken is shown in drawing 1 (lane 2) after ethidium bromide dyeing. In the sample extracted from the conventional method shown in the comparative example 1, it was checked that genomic DNA mixes so much so that clearly from drawing 1 (lane 2).

[0031]

[Effect of the Invention]In this invention, a suitable solution and nucleic acid unity solid phase are used under an acid condition.

Therefore, by making RNA contained in living thing material adsorb specifically, and using a still more suitable eluate, without needing complicated post-processing operation, these RNA is collected simple and extraction refining can be carried out.

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## DESCRIPTION OF DRAWINGS

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[Brief Description of the Drawings]
[Drawing 1]It is a photograph replaced with the Drawings in which the agarose-gel-electrophoresis pattern of RNA by which extraction refining was carried out from the cultured cell is shown with the method and conventional method of this invention.
[Drawing 2]It is a photograph replaced with the Drawings in which the agarose-gel-electrophoresis pattern of the RT-PCR amplification products of RNA by which extraction refining was carried out from the cultured cell is shown by the method of this invention.
[Drawing 3]It is a photograph replaced with the Drawings in which the agarose-gel-electrophoresis pattern of the RT-PCR amplification products of RNA by which extraction refining was carried out from the HCV positive blood serum is shown by the method of this invention.

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[Translation done.]